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<b>(21) International Application Number:</b> PCT/GB94/02092 <b>(22) International Filing Date:</b> 26 September 1994 (26.09.94) <b>(30) Priority Data:</b> 9319772.1 24 September 1993 (24.09.93) GB <b>(71) Applicants (for all designated States except US):</b> THEREXSYS LIMITED [GB/GB]; Suite 1-8, The Science Park, University of Keele, Keele, Staffordshire ST5 5SP (GB). MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CRAIG, Roger, Kingdon [GB/GB]; Jubilee House Farm, Spen Green, Smallwood, Sandbach, Cheshire CW11 0XA (GB). GROSVELD, Franklin, Gerardus [NL/NL]; Jacobus Van Vessemsingel 9, NL-3065 NL Rotterdam (NL). DZIERZAK, Elaine [US/GB]; 40 Patshill Road, London NW5 2JY (GB). ABRAHAM, David [GB/GB]; 12 Cedar Close, Ditton, Maidstone, Kent ME20 6EN (GB). <b>(74) Agent:</b> HALLYBONE, Huw, George; Carpmals & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ). <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EXPRESSION OF VIRAL DECOY PROTEINS UNDER THE CONTROL OF A LOCUS CONTROL REGION AND USES THEREOF		
<b>(57) Abstract</b> <p>There is described a recombinant nucleic acid vector for the delivery of nucleic acid to a host organism comprising a transcription unit encoding a transdominant negative mutant of a viral gene product which has been selected substantially to avoid a negative biological effect in the host under the control of a DNA sequence active in cells normally infected by a virus which is effective to confer constitutive tissue-specific, integration site-independent, copy-number dependent expression of the transcription unit.</p>		

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EXPRESSION OF VIRAL DECOY PROTEINS UNDER THE CONTROL OF A LOCUS  
CONTROL REGION AND USES THEREOF

The present invention relates to an agent for anti-viral therapy which possesses a protective effect when  
5 administered to healthy individuals. In particular, the agent of the invention may be a mutant HIV gene product.

**Introduction**

10 Human immunodeficiency virus (HIV) has been identified as the etiological agent in human acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984). Conventional therapeutic strategies have concentrated on antiviral drugs such as AZT and on the  
15 development of preventive vaccines. However, the intracellular immunisation approach (Baltimore, 1988) has lead to the development of molecular strategies for the inhibition of HIV replication (Malim et al., 1989, Trono et al., 1989, Sczakiel et al., 1991, Sullenger et al., 1990).

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Molecular systems for *in vivo* cell specific therapy have been described whereby a gene encoding a toxic product can be controlled in its expression by regulatory regions of genes active only in particular cells.

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Initial studies on cell-specific ablation therapy have utilised cytotoxic agents such as diphtheria toxin A or ricin A chain genes under the control of lens (Breitman et al., 1987; Landel et al., 1988) or pituitary (Behringer et  
30 al., 1988) specific promoters. After microinjection into mouse embryos and the production of transgenic animals, these constructs resulted in the destruction of either lens or pituitary cells. However, when associated with leaky promoter elements, these toxin genes are unsuitable for  
35 somatic therapy because of the constitutive cell lethality and the extreme sensitivity of mammalian cells to diphtheria and ricin toxins.

A more versatile toxin-encoding gene for potential use in human ablative therapy has been described (Borelli et al., 1988). The Herpes Simplex Virus type 1 thymidine kinase (tk) gene product is a conditional cell lethal and has been  
5 shown to be toxic to mammalian cells only in the presence of nucleoside analogues such as acyclovir (ACV) or gancyclovir (GCV). These analogues kill actively cycling cells because they possess high affinity for the tk gene product with little or no affinity for endogenous mammalian tk. Model  
10 systems have demonstrated *in vivo* lymphocyte specific lethality by anti-herpetic drug treatment of tk transgenic mice (Borelli et al., 1988, Heyman et al., 1989). Specificity of conditional toxicity is due to lymphoid specific transcriptional control elements and quantitative  
15 flexibility is inherent within the levels of tk transgene expression and/or administered drug dose. Upon withdrawal of the drug in these studies, mature lymphocytes are restored to normal numbers. Thus, the *in vivo* ablative system is regenerative, reversible and does not affect stem  
20 cells.

Even advanced ablative systems, however, have their disadvantages in antiviral therapy. In particular, the rationale of the system is flawed in that it relies on the  
25 destruction of virally infected cells. Although this prevents viral replication, the pathogenic effect of the virus, the destruction of T-lymphocytes in the case of HIV, is actually promoted. Therefore, ablative systems are unlikely to be applicable to cases of established infection  
30 and must rely on reaching substantially all cells infected by the virus.

Another potential system for use in anti-HIV therapy involves the expression in cells susceptible to HIV  
35 infection of a decoy gene.

Decoy genes encode proteins which act as antagonists to natural proteins involved in the replication of the HIV

virus. For example, a decoy gene may encode a defective mutant of a transactivator protein which is capable of binding to the transactivator-responsive site on the host or viral genome, yet is incapable of activating transcription.

5

Transdominant mutations have been reported in a number of viral transactivators which abolish or attenuate the ability of the wild-type protein to transactivate the target gene. Examples include transdominant mutations of E1A (Glen et al., 1987), tax (Wachsman et al., 1987) and VM65 (Friedman et al., 1988). Similar mutations in HIV genes have been described for the Tat transactivator (Pearson et al., 1990) and the Rev transactivator (Bevac et al, 1992; Malim et al., 1992).

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Expression of such mutant proteins in a HIV-infected cell line leads to competition with the natural transactivator and resultant loss of transactivating activity. See, for example, International patent application WO 9014427 (Sandoz). A potential disadvantage of the use of decoy gene approaches is that when a decoy is expressed in the absence of the infecting virus a negative biological effect may be exerted on the host. For example, a host immune response may result from the production of the decoy gene product, leading to destruction of the host cell by, for example, cytotoxic T-lymphocytes (CTL). Furthermore, it must be borne in mind that decoy proteins are derived from biologically active gene products of pathogenic organisms and may therefore exert a deleterious effect on the host. For example, certain allelic variants of the HIV nef gene product have been shown to downregulate CD4 expression on thymocytes and to reduce the numbers of CD4<sup>+</sup> thymocytes in transgenic mice (see our copending U.K. Patent Application No. 9305759.4 and Guy et al., 1990).

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In order to avoid these disadvantages it has been suggested that transcription and expression of decoy gene products should be restricted to cells actually infected by HIV, for

example by using a transactivatable expression system for decoy expression which is transactivated by an HIV gene product (see WO 9011359).

- 5 However, because the decoy gene product is only expressed in cells after HIV infection, a considerable excess of decoy gene product is required in order to successfully arrest viral replication, since the virus has an effective head start. The production of large amounts of decoy in infected
- 10 cells is likely to give rise to the aforementioned negative biological effects against such cells, leading to death or incapacitation of infected cells and the concomitant disadvantages of ablative systems discussed above.
- 15 An alternative approach, which has not been proposed in the prior art, would be to ensure constitutive expression of decoy proteins which do not give rise to a negative biological effect in cells susceptible to HIV infection.
- 20 Locus control regions (LCRs) are elements which confer position-independent, copy number-dependent expression of genes in gene transfer approaches. They have also been shown to permit high levels of expression of cloned genes and to possess tissue-specific properties. First discovered
- 25 in globin genes (Grosveld et al, 1987) these elements are believed to direct the creation of independent regulatory domains within the chromatin structure of cell genomes, thereby ensuring the activity of a co-transferred gene.
- 30 A number of LCRs other than those for globin genes have been described, for example in the CD2 gene in T-lymphocytes (Greaves et al., 1989) and the lysozyme gene in macrophages (Bonifer et al., 1990) and B-cells (see European patent application 460042).

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The targets of HIV infection are primarily CD4<sup>+</sup> T-lymphocytes, but also include macrophages and, dendritic cells which are related to macrophages and of importance in

initiating an immune response (reviewed in McCune, 1991). These cells share very few common features except for being derived from common hematopoietic stem cells and susceptibility of HIV infection. Hematopoietic stem cells  
5 are not infected by HIV (Molina et al., 1990; David et al., 1991).

We have now determined that by inserting a transcription unit encoding a decoy gene under the control of the CD2 LCR  
10 into stem cells, constitutive expression of the decoy may be achieved in T-lymphocytes in transgenic mice and passed through the germ line, without disadvantageous effects on the host, by selection of decoy genes having low deleterious properties.

15 According to a first aspect of the invention, therefore, there is provided a recombinant nucleic acid vector for the delivery of nucleic acid to a host organism comprising a transcription unit encoding a transdominant negative mutant  
20 of a viral gene product which has been selected substantially to avoid a negative biological effect under the control of a DNA sequence active in cells normally infected by a virus which is effective to confer constitutive tissue-specific, integration site independent,  
25 copy-number dependent expression of the transcription unit.

By "substantially avoid a negative biological effect" it is intended to denote that the decoy used in the present invention has been selected or specifically modified to give  
30 rise to a negligible adverse effect on host cells, which may safely be disregarded in therapeutic situations, or, preferably, no adverse effect at all. The negative biological effect may be a biological impairment of cell function or the raising of a CTL response, as set out above,  
35 or both.

By "biological impairment of cell function" it is intended to denote biological effects on host cells, such as

downregulation of CD4, which may have a negative effect on the patient. In some cases the host cells may be affected by the decoy in a manner similar to the pathologic activity of the virus. In other cases, the decoy proteins may have  
5 effects not normally associated with viral infection but which are undesirable when associated with a constitutively expressed foreign protein.

For example, where the virus is HIV, the decoy is selected to  
10 avoid, *inter alia*, an impairment of immune function in the host.

Impairment of immune function has been demonstrated *in vitro* for certain allelic variants of nef (Guy et al., 1990).  
15 These results are reinforced *in vivo* in our copending U.K. Patent Application No. 9305759.4, which shows that nef expression may play a role in CD4 downregulation and in decreasing the numbers of CD4<sup>+</sup> T-cells in transgenic mice. CD4 downregulation and loss of CD4<sup>+</sup> T-cells is one of the  
20 pathogenic features associated with HIV infection and AIDS.

CTL response to SIV proteins has been observed in SIV-infected macaques (Venet et al., 1992) and in human retroviral infection (Kannagi et al., 1983; Mitsuya et al.,  
25 1983; Autran et al., 1991; Nixon and McMichael, 1991). CTL responses to Nef antigens and Rev antigens are strong. CTL responses to Tat antigens are, however, rare and thought to be very weak (Lamhamedi-Cherradi et al., 1992).

We have shown that the HIV tat gene product does not impair  
30 cell function in transgenic mice. The tat gene product, therefore, appears to exert little or no impairment of cell function, while at the same time is of very low CTL-inducing activity. Preferably, therefore, the invention comprises the constitutive expression of a Tat decoy.

35

The invention further provides the use of other natural decoy gene products which possess the desired characteristics displayed by tat, namely the absence of



substantial negative biological effects.

Alternatively, the invention provides for the use of a decoy gene product which has been specifically mutated to reduce the incidence and strength of negative biological responses thereto. This may be achieved, for example, by mutation or deletion of certain domains of a decoy gene product. For example, the cell-impairing effects of a decoy gene product may be reduced or eliminated by the introduction of point mutations in the gene (see Guy et al., 1990).

For example, we have shown that, in transgenic mice, expression of the Tat gene product under the control of the CD2 LCR gives rise to a three-fold increase in the levels of mRNA encoding certain cytokines, namely TGF- $\beta$ , IL4 receptor and TNF- $\beta$ .

In contrast, when a mutated Tat gene product is used comprising a point mutation which abolishes its effector function, cytokine mRNA levels are not affected. advantageously, therefore, a mutated Tat gene product is used in the present invention.

CTL response to a protein may be modified, either by the introduction of mutations at certain residues involved in binding to the presenting HLA molecules or interacting with the T-cell receptors (see Choppin et al., 1992; 1991a, b; Gotch et al., 1988). Furthermore, it is possible to modify a protein to reduce its rate of degradation by the cell and thereby lower the incidence of presentation of antigens derived from the protein (Bachmair et al., 1986; Townsend et al., 1988).

It has been shown that HIV naturally mutates to avoid CTL responses *in vivo* and that the sites of amino acid variation tend to be conserved, at least to a certain extent, in different patients (Phillips et al., 1991). Preferably, therefore, the decoy of the invention may be mutated in

accordance with naturally-derived HIV isolates which display a reduced CTL response.

However, it should be pointed out that CTL responses will vary between individuals due to variation in the antigen-presenting molecules present. Therefore, although some general mutations may be carried out to reduce CTL response caused by common HLA types, it remains possible that certain individuals may show a CTL response even to decoys which are believed to be of low CTL-inducing activity. In this case, the invention provides means to mutate the decoy on an individual basis in order to reduce or eliminate the CTL response in that individual.

By "transdominant negative mutant" it is intended to refer to a gene product which is rendered functionally transdominant over its viral analogue and is effective to block the activity of the viral analogue. Therefore, the term is to be interpreted functionally, and comprises mutants in the normal sense of the term, having an altered amino acid sequence, as well as mutants which are alternatively processed or spliced, and mutants which differ from the wild-type protein in patterns of expression. For example, the Nef gene product of HIV is known to be a transdominant inhibitor when expressed in excess. Therefore, an overproduced Nef protein is included in the term "transdominant negative mutant".

Constitutive expression confers a particular advantage of the invention, that is that the presence of the decoy in healthy cells effectively prevents the infecting virus from becoming established. If the decoy gene is only activated after infection by the virus, there is the possibility that the virus may become established before the decoy is able to exert any significant anti-viral effect

The DNA sequence controlling the transcription unit of the vector of the invention is preferably a Locus Control Region

(LCR). A number of LCRs have been described in the art and the selection of an appropriate LCR is within the capabilities of a person skilled in the art. In the case of the treatment of HIV infections, however, the use of the CD2  
5 and the macrophage-specific lysozyme LCR is preferred. Both T-cells, in which the CD2 LCR is active, and macrophages are targets for HIV infection.

In addition to the LCR, the vector of the invention is  
10 equipped with a promoter which is constitutively active in the target tissue type. For example, if the cells to be targeted are T-cells, the CD2 promoter may be used. The promoter, however, may be active in cells other than the target tissue. In such a case, high-level expression in  
15 non-target cells is unlikely, because the LCR is inactive in these cells. Even if a certain amount of non-specific expression cells does occur, such expression will not be harmful as the gene product is selected to avoid negative biological effects. In any event, non-specific expression  
20 can be minimised by the use of efficient vector targeting techniques to deliver the vector of the invention.

The nucleic acid vector may be any vector capable of delivering nucleic acid to a cell. For example, the vector may be a plasmid, a virus or a linear DNA fragment. The  
25 vector may be naked, complexed with proteins or packaged in a delivery system such as a liposome, virosome, or receptor mediated complex.

The vector of the invention is preferably for use in the  
30 transfection of stem cells. Therefrom stems a further advantage of the invention, that is that the decoy is expressed in all cells of a particular lineage.

When the vector encodes an HIV decoy, the stem cells may be  
35 hematopoietic stem cells.

Alternatively, T-cells may be targeted directly. The targeting of T-cells is desirable, for example, in the case

where HIV infection is already established but the virus has not yet spread to the peripheral T-cell population. In this instance, such cells may be effectively protected from viral infection.

5

A number of protocols for the transfection of stem cells and T-cells are known in the art. Some involve the isolation of stem cells from total cell populations, as described in, for example, European patent applications 0 455 482 and 0 451

10 611.

An improved process for the transfection of stem cells and T-cells is described in our copending U.K. Patent Application No. 9317380.5, the disclosure of which is  
15 incorporated herein by reference.

The host organism may be a mammal, insect, fish, plant or any other organism which it is desired to protect from viral disease. Preferably, the host organism is man.

20

Where the decoy is a Tat decoy, the decoy may be prepared following any of the protocols known in the art. For example, the method of Pearson *et al.* (1990) may be used to  
25 generate deletion mutants of Tat which lack the transactivating function but retain the ability to bind to the tat region. Such deletion mutants may be tested for decoy activity as described in Pearson *et al.* or according to the methods set forth in our copending U.K. Patent  
30 Application No. 9305759.4 as well as International Patent Application WO90/14427.

The potential toxicity of any such mutants may be tested by the methods described hereinbelow. Should such mutants  
35 prove to give rise to a CTL response in a patient, they may be further mutated to reduce this response, in accordance with methods known in the art.

The decoy gene may be derived from any virus which gives rise to infection in man or other organisms, including plants. Especially preferred, however, are HIV decoys such as Tat, Rev or Nef decoys.

5

The decoy may be derived from the same virus as it is intended to combat with the vector of the invention. However, it is envisaged that decoys derived from viruses other than one it is intended to treat may be used. For  
10 example, it has been noted that an HTLV1 Rex decoy is active in suppressing HIV1 Rev function (Bohnlein et al., 1991). Furthermore, it is envisaged that entirely artificial decoy genes encoding specialised decoy proteins may be designed. For example, an artificial decoy gene may be designed which  
15 encodes the tar-binding domain only of the HIV Tat transactivator, or an analogue of the tar-binding domain which effectively competes for tar binding with wild type Tat.

20 The vector of the invention is preferably for use in the transfection of a patient's cells *in vivo* or *ex vivo*, for the treatment of a viral disease. According to a second aspect of the invention, therefore, there is provided a vector according to the first aspect of the invention for  
25 use in therapy.

By "therapy", it is intended to denote both the prevention and the attenuation or elimination of viral infection. As set out hereinbefore, it is preferred that the vectors of  
30 the invention be used for the prophylaxis of viral infections because it is believed that it may be important to avoid establishment of the viral infection in the host. However, especially in the early stages of a viral infection, it is envisaged that the vectors of the invention  
35 may have a conventional therapeutic application effective to attenuate and eventually eliminate the viral infection.

Preferably, the vector of the invention is used for the

treatment of stem cells or T-cells ex vivo. Accordingly, in a third aspect of the present invention there is provided a vector according to the first aspect of the invention for use in the treatment of stem cells or T-cells ex vivo.

5

Stem cells or T-cells may be isolated according to procedures described in the prior art, as set out hereinbefore. Alternatively, as is preferred, stem cells or T-cells may be targeted using an efficient targeted  
10 transfection technique, such as that described in our copending U.K. Patent Application No. 9317380.5. Using such a technique, it is possible to transfect stem cells or T-cells in whole blood obtained from patients with extremely high efficiency.

15

According to a further aspect of the invention, there is provided a method for treating or preventing a viral infection comprising the steps of:

- 20 a) removing a cell from the body of a patient;
- b) transfecting the cell with a vector according to the first aspect of the invention; and
- 25 c) returning the cell to the body of the patient.

Preferably, the cell is a stem cell. For example, the cell is a haematopoietic stem cell. Alternatively, the cell may be a T-cell.

30

Haematopoietic stem cells and T-cells are easily removed from the body of a patient, for example from cord blood, peripheral blood or bone marrow aspirate.

35 Transfection of stem cells may be accomplished by any of the protocols cited hereinbefore.

The transfected stem cells, once returned to the body of the

patient, will divide in the usual manner and populate the patient with cell lineages carrying the heterologous gene comprised in the vector of the invention. The cell lineages thus derived will possess the antiviral capabilities  
5 conferred by the heterologous gene.

According to a still further aspect of the invention, there is provided the use of a vector according to the invention in the manufacture of a composition for use in the treatment  
10 or prophylaxis of a viral disease.

Preferably, the composition comprises the vector of the invention in a suitable buffer for use in the transfection of cells either *in vivo* or *ex vivo*. When used *in vivo*, the  
15 buffer will consist essentially of pharmaceutically acceptable excipients, diluents or carriers. For use *ex vivo*, the nature of the buffer will be determined by the transfection protocol being employed. For example, if the method described in our copending U.K. Patent Application  
20 No. 9317380.5 is to be used, the buffer as described therein is used.

In a still further aspect of the invention, there is provided a cell comprising a transcription unit encoded by  
25 the vector of the invention. Preferably, the cell is a stem cell and advantageously it is a haematopoietic stem cell. Alternatively, the cell may be a T-cell.

The invention further provides a method for the treatment or  
30 prevention of a viral infection comprising administering to a patient a pharmaceutically effective amount of a composition comprising the vector of the invention in admixture with a pharmaceutically acceptable excipient, diluent or carrier.

35

The invention will now be described for the purpose of illustration only in the following examples, with reference to the Figures, in which:

Figure 1 is a diagrammatic representation of the structure of the CD2-Tat transgene;

Figure 2 shows the identification of the Tat DNA in transgenic mice carrying the CD2-Tat transgene;

Figures 3 A and B show the identification of Tat RNA in transgenic mice carrying the CD2-Tat transgene;

Figure 4 shows a FACS analysis of thymus tissue from transgenic and non-transgenic mice;

Figure 5 shows a FACS analysis of spleen and lymph node tissues from transgenic and non-transgenic mice;

15

Figure 6 shows the impact of the presence of the Tat transgene on cytokine gene expression in transgenic mice;

Figure 7 is a slot blot showing the generation of transgenic mice carrying a mutated Tat transgene in which Tyr 47 has been mutated to Ala; and

20

Figure 8 is a slot blot which demonstrates that mutant Tat has no effect on the expression of the TNF- $\beta$  gene.

25

#### METHODS

##### **CD2-Tat mice**

A DNA fragment comprising the Tat coding sequence (see Figure 1) was ligated into a unique EcoR1 site in the first exon of the CD2 gene in the p2629 CD2 expression plasmid, which was obtained from Dr. D. Kioussis, NIMR, Mill Hill, Great Britain. A 4.5 kb fragment containing the CD2 LCR was isolated from p2694 (also obtained from Dr. Kioussis) and ligated into the unique Bam H1 - Not 1 sites of p 2629. The 12 kb Sal 1 - Not 1 fragment comprising the CD2-Tat construct was then excised and microinjected into single-

30  
35



cell mouse embryos as previously described (Grosveld et al., 1987). Positive founder animals were bred with CBA x C57 BL/10 mice and lines maintained as heterozygotes.

## 5 DNA and Expression Analysis

Tail DNA (10 $\mu$ g) from founder animals was analysed by Southern blot analysis after digestion with HindIII or Asp718. DNA was run on a 1% agarose/Tris-acetate, EDTA gel, 10 blotted onto nitrocellulose and probed with a randomly primed BamHI-SmaI Tat fragment. A 1.2 Kb Thy-1.2 fragment was used as a loading control probe.

Appriopriate amounts of pCD2Tat spiked in 10  $\mu$ g genomic DNA 15 were used as a copy number controls. Quantitation was performed on the Molecular Dynamics PhosphorImager.

RNA was prepared using the lithium chloride/urea method (Fraser et al., 1990). For Northern blot analysis (Sambrook 20 et al., 1989) 10 $\mu$ g of RNA was run on a 1% formaldehyde gel, blotted onto nitrocellulose and probed with a 800 bp BamHI-SmaI nef fragment from pTG1147. For RNA slot blots (Sambrook et al., 1989) 5 $\mu$ g of RNA was blotted onto nitrocellulose and probed as above. RNA from the Nef 25 producing CRIP L producer cell line (Schwartz et al., 1992) was used as a positive control.

### Example 1

## 30 Expression of CD2-Tat in Transgenic Mice

Exon 1 (encoding aa 1-72) of the HIV-1 TAT gene was inserted downstream of the transcriptional start site in the first exon of the human CD2 gene (Figures 1 and 2). A stop codon 35 was constructed in the sequence of human CD2 exon 2 so as to eliminate the production of CD2 prtein. The human CD2 LCR element was ligated to the 3' end of the construct. A SalI-NotI fragment was injected into fertilized mouse eggs. At

least three transgenic lines were created. Line C (2 on figure 3A) contains 70 copies and line E (4 on figure 3A) contains 40 copies.

5 S1 nuclease RNA protection was performed on various tissues from a transgenic and a non-transgenic mouse using a TAT exon 1 probe. As shown in figure 3B only thymus expressed TAT highly. Spleen expressed Tat only to low levels. No expression was observed in the kidney or liver of the  
10 transgenic mouse or in any of the tissues of the control non-transgenic.

In order to determine whether the CD4 and CD8 T cells subsets are affected by the overexpression of HIV-TAT,  
15 antibody staining and FACS analysis was performed on thymocytes, spleen and lymph node cells from CD2-TAT transgenic mice (Figures 4 and 5). Single cell suspensions were prepared from the tissues of line C and line E transgenic mice (samples 3 and 4 respectively) and their  
20 non-transgenic littermates (samples 1 and 2). PE labeled CD8 and FITC labeled CD4 antibodies were incubated with the cells and FACS analysis performed. As shown in the contour plots, no changes in the percentage of double negative, double positive or single positive subsets were found in the  
25 thymus of transgenic mice. Furthermore, no changes in the percentages of CD4 or CD8 single positive subsets were found in spleen or lymph nodes of the transgenic mice when compared to non-transgenics. Thus, high level expression of HIV-TAT does not affect subset distribution in vivo in the  
30 lymphoid organs.

TAT induced transcriptional upregulation of  $\text{TNF-}\beta$  leads to overproduction of functional  $\text{TNF-}\beta$  as measured by cytotoxicity.

35

Northern blot analysis of RNA from TAT transgenic lines C and E demonstrated an increase of 2.3 fold in  $\text{TNF}\beta$  transcription (Figure 6A). In order to test whether this

resulted in increased TNF- $\beta$  protein production, we performed cytotoxicity assays with cell lysates of T cells from TAT transgenic mice (C+1, C+2, E+1 and E+2) and non-transgenic littermates (C- and E-). Active equivalents of TNF- $\beta$  protein in each sample was quantitated against a known TNF- $\beta$  protein standard (in units). As shown in Figure 6B, all of four transgenic mice produced significantly higher levels of TNF- $\beta$  (2-4 fold) as compared to the non-transgenic controls.

10

**Cytokine gene expression is affected by the pressure of HIV-TAT.**

Northern blot analysis was performed on RNA from CD2-TAT transgenic mice to test for quantitative differences in cytokine gene expression (Figure 7). Total mRNA was prepared from thymocytes of line C transgenic mice (C+1 and C+2) and a non-transgenic littermate (C-) and from thymocytes of line E transgenic mice (E+1 and E+2) and a non-transgenic littermate (E-). 10  $\mu$ g of RNA was loaded per lane on a formaldehyde agarose gel. RNA was transferred onto a filter and hybridized with a  $\beta$ -actin probe as an RNA quantitation control and a TAT probe for verification of transgene expression. The filter was rehybridized several times with probes for cytokine genes TGF- $\beta$ , IL-4R, TNF- $\beta$  and TNF- $\alpha$ . Autoradiograms of the Northern blot demonstrate an increase in expression of TGF- $\beta$ , IL-4R and TNF- $\beta$  gene expression in the TAT transgenic mice. However, hybridization signal with the TNF- $\alpha$  probe suggests no change or a decrease in TNF- $\alpha$  gene expression in the Tat positive mice.

The results of this Northern blot were quantitated on a PhosphorImager and fold increases or decreases were calculated. As shown in Table 1, when signal was normalized against the  $\beta$ -actin quantitation control, TGF- $\beta$ , IL-4R and TGF- $\beta$  steady state mRNA was increased in the transgenic mice. TGF- $\beta$  levels increased on average 2.8 fold, IL-4R

levels increased 3.5 fold and TNF- $\beta$  levels increased 2.3 fold. TNF- $\alpha$  levels decrease by about 25%. Thus, the expression of TAT in thymocytes has an effect on cytokine gene expression.

5

Table 1

**Quantitation of Cytokine RNAs in CD2-TAT Transgenic Mice**

10	Transgenic line Mouse	Line C		Line E	
		1	2	1	2
	TGF- $\beta$	2.20	3.26	2.74	2.88
	IL-4R	3.40	2.76	4.03	3.76
15	TNF- $\beta$	2.29	2.32	2.20	2.22
	TNF- $\alpha$	0.81	0.67	0.74	0.85

Fold RNA change as compared to non-transgenic littermates.

20

Example 2

**Expression of CD2-TAT (47ala) in transgenic mice.**

25 A 12kb SalI-NotI fragment containing the CD2 promoter and LCR element and exon 1 (encoding aal-72) of a mutant form of the HIV-1 TAT gene in which amino acid 47 had been converted from a tyrosine into an alanine (47 Tyr---> Ala), was injected into fertilized mouse eggs. As shown in figure 7,  
30 three transgenic founders were created (Lines A6, A7 and A8). In comparison with transgene copy number controls from 0 to 50 (Lane B), these founders contained between 8 and 25 copies of the transgene.

35 In order to determine whether thymocyte T cell subsets are affected by the expression of the mutant form of the HIV-TAT (47ala), antibody staining and FACS analysis was performed on thymocytes from CD2-TAT (47ala) transgenic

mice. Single cell suspensions were prepared from mouse A6 and a non-transgenic animal, A4. PE-labelled CD8 and FITC-labelled CD4 antibodies were incubated with the cells and FACS analysis was performed. As shown in Table 2, no changes were found in the total number of thymocytes derived from either transgenic or non-transgenic mice. Furthermore, no difference was observed in the relative percentages of double negative (DN), double positive (DP), or single positive (SP) CD4 or CD8 cells in the thymus from the transgenic compared with the non-transgenic mouse.

Table 2

#### Thymic T cell Subsets in CD2-TAT (47ala) Transgenic Mice

	DN	DP	SP	SP
Total (CD4-CD8-) (CD4+/CD8+) (CD4+) (CD8+)				
T-Cells				
x10 <sup>-8</sup>				
Non-Transgenic	2.5	4.1	80.7	11.5 3.7
Transgenic	2.2	7.4	75.9	12.6 4.1

25

Cytokine gene expression (TNF- $\beta$ ) is not affected by the presence of the mutant HIV-TAT (47ala)

RNA slot blot analysis was performed on RNA from CD2-TAT (47ala) transgenic mice to test for quantitative differences in cytokine gene expression (Figure 8). Total RNA was prepared from the thymocytes of CD2-TAT (47ala) transgenic mice A.6 and A.7, two non transgenic controls A.4 and A.5, and from two transgenic mice, one from line C(c.1) and one from line E(E.1) harbouring the wild type HIV-TAT. Duplicated 10 $\mu$ g RNA samples were denatured and loaded onto the filter and hybridized with a  $\beta$ -actin probe as an RNA quantitation control, and then with a probe specific for the

cytokine TNF- $\beta$ . Autoradiographs of the RNA blots demonstrate that while an increase in expression of TNF- $\beta$  is seen in transgenic mice containing the wild type HIV-TAT, no increase in expression of TNF- $\beta$  is observed in mice  
5 containing the mutant HIV-TAT (47ala).

The results of this RNA slot blot were quantitated on a phosphorImager and the fold changes in cytokine gene expression determined. As shown in Table 3, when the signals  
10 are normalized against the internal quantitation control  $\beta$ -actin, although TNF- $\beta$  steady state mRNA was elevated in lines C and E, there was no increase in expression of this cytokine in the mutant HIV-TAT (47ala) transgenic mice. Thus, the expression of the mutant HIV-TAT (47ala) has no  
15 effect on TNF- $\beta$  cytokine expression.

TABLE 3

20 **Quantitation of cytokine (TNF) $\beta$  RNA in HIV-TAT (47ala) transgenic mice**

25	Transgenic mouse	A.6	A.7	C.1	E.1
	TNF- $\beta$	0.97	0.89	1.9	2.4

Fold RNA change as compared with non-transgenic mice.

### References

- Autran et al., (1991) J. Acquir. Immune Defic. Syndr., 2, 398-403
- 5
- Bachmair et al., (1986) Science, 234, 179
- Baltimore, (1988) PNAS, 85, 9753-9757
- Barre-Sinoussi et al., (1983) Science, 220, 868-870
- Behringer et al., (1988) Genes and Dev., 2, 453-461
- 10 Bevac et al., (1992) PNAS, 87, 9870-9874
- Bohnlein et al., (1991) J. Virol., 65, 81-88
- Bonifer et al., (1990) EMBO J., 9, 2843-2848
- Borelli et al., (1988) PNAS, 85, 7572-7576
- Breitman et al., (1987) Science, 238, 1563-1565
- 15
- Choppin et al., (1991a) J. Immunol., 147, 569-574
- Choppin et al., (1991b) J. Immunol., 147, 575-583
- Choppin et al., (1992) Crit. Rev. Immunol., 12, 1-16
- 20 David et al., (1991) J. Viral., 65, 1985-1990
- Friedman et al., (1988) Nature, 335, 452-454
- Gallo et al., (1984) Science, 224, 500-503
- 25 Glen et al., (1987) Mol. Cell. Biol., 4, 1004-1010
- Gotch et al., (1990) Virology, 176, 413-425
- Greaves et al., (1989) Cell, 56, 979-986
- Grosveld et al., (1987) Cell, 51, 975-985
- Guy et al., (1988) J. Exp. Med., 168, 2045-2057
- 30
- Heyman et al., (1989) PNAS, 86, 2698-2702
- Kannagi et al., (1983) J. Immunol., 130, 2942-2946
- 35 Lamhamedi-Cherradi et al., (1992) AIDS, 6, 1249-1258
- Landel et al., (1988) Genes and Dev., 2, 1168-1178

- Malim et al., (1989) Cell, 58, 205-214
- Malim et al., (1992) J. Exp. Med., 176, 1197-1201
- McCune, (1991) Cell, 64, 351-363
- McMichael et al., (1977) Nature, 270, 524
- 5 Mitsuya et al., (1983) J. Exp. Med., 158, 944-1041
- Molina et al., (1990) Blood, 76, 2476-2482
- Morrison et al., (1992) Eur. J. Immunol., 22, 903-907
- Nixon and McMichael, (1991) AIDS, 5, 1049-1059
- 10 Pearson et al., (1990) PNAS, 87, 5079-5083
- Phillips et al., (1991) Nature, 354
- Sambrook et al., (1989) Molecular Cloning: A Laboratory
- 15 Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schwartz et al., (1992) AIDS Research Hum. Retroviruses, 8, 545-551
- Sczackiel et al, (1991) J. Viral., 65, 468-472
- 20 Sullenger et al., (1990) Cell, 63, 601-608
- Townsend et al., (1988) J. Exp. Med., 168, 1211-1224
- Trono et al., (1989) Cell, 59, 113-120
- 25 Venet et al., (1992) J. Immunol., 148, 2899-2908
- Wacksman et al., (1987) Science, 235, 674-677



CLAIMS:

1. A recombinant nucleic acid vector for the delivery of nucleic acid to a host organism comprising a transcription  
5 unit encoding a transdominant negative mutant of a viral gene product which has been selected substantially to avoid a negative biological effect in the host under the control of a DNA sequence active in cells normally infected by a virus which is effective to confer constitutive tissue-  
10 specific, integration site-independent, copy-number dependent expression of the transcription unit.
2. A recombinant nucleic acid vector according to claim 1 encoding a viral gene product which has been selected  
15 substantially to avoid a CTL response in the host.
3. A recombinant nucleic acid vector according to claim 1 or claim 2 encoding a viral gene product which has been selected substantially to avoid immune impairment in the  
20 host.
4. A recombinant nucleic acid vector according to any preceding claim wherein the DNA sequence controlling the transcription unit comprises a Locus Control Region (LCR).  
25
5. A recombinant nucleic acid vector according to any preceding claim wherein the DNA sequence controlling the transcription unit comprises a constitutively active  
30 promoter.
6. A recombinant nucleic acid vector according to any preceding claim which is specific for a target cell.
- 35 7. A recombinant nucleic acid vector according to claim 4 wherein the target cell is a haematopoietic stem cell.

8. A recombinant nucleic acid vector according to claim 4 wherein the target cell is a T-cell.
9. A recombinant nucleic acid vector according to claim 5 4 wherein the target cell is a macrophage.
10. A recombinant nucleic acid vector according to any preceding claim wherein the transcription unit encodes a transdominant negative mutant of an HIV gene product.
- 10 11. A recombinant nucleic acid vector according to claim 8 wherein the transcription unit encodes a transdominant negative mutant of the HIV Tat gene product.
- 15 12. A recombinant nucleic acid vector according to any preceding claim wherein the transcription unit encodes a transdominant negative mutant which is further mutated to reduce any negative biological effect.
- 20 13. A recombinant nucleic acid vector according to claim 12 wherein the transcription unit encodes a transdominant negative mutant which has been modified to reduce or eliminate effects on cytokine gene expression.
- 25 14. A nucleic acid vector according to any preceding claim for use in therapy.
15. A nucleic acid vector according to claim 14 for use in the treatment of a stem cell, a T-cell or a macrophage in vivo.
- 30 16. A nucleic acid vector according to claim 14 for use in the treatment of a stem cell, a T-cell or a macrophage ex vivo.
- 35 17. A method for treating or preventing a viral infection comprising the steps of:

- a) removing a cell from the body of a patient;
- b) transfecting the cell with a vector according to any one of claims 1 to 8; and

5

- c) returning the cell to the body of the patient.

18. A method for treating or preventing a viral infection comprising administering to a patient a pharmaceutically effective dose of a composition comprising the vector of any one of claims 1 to 13 in admixture with a pharmaceutically acceptable excipient, diluent or carrier.

19. The use of a vector according to any one of claims 1 to 13 in the manufacture of a composition for use in the treatment or prophylaxis of a viral disease.

20. A cell comprising the transcription unit encoded by the vector of any one of claims 1 to 13.

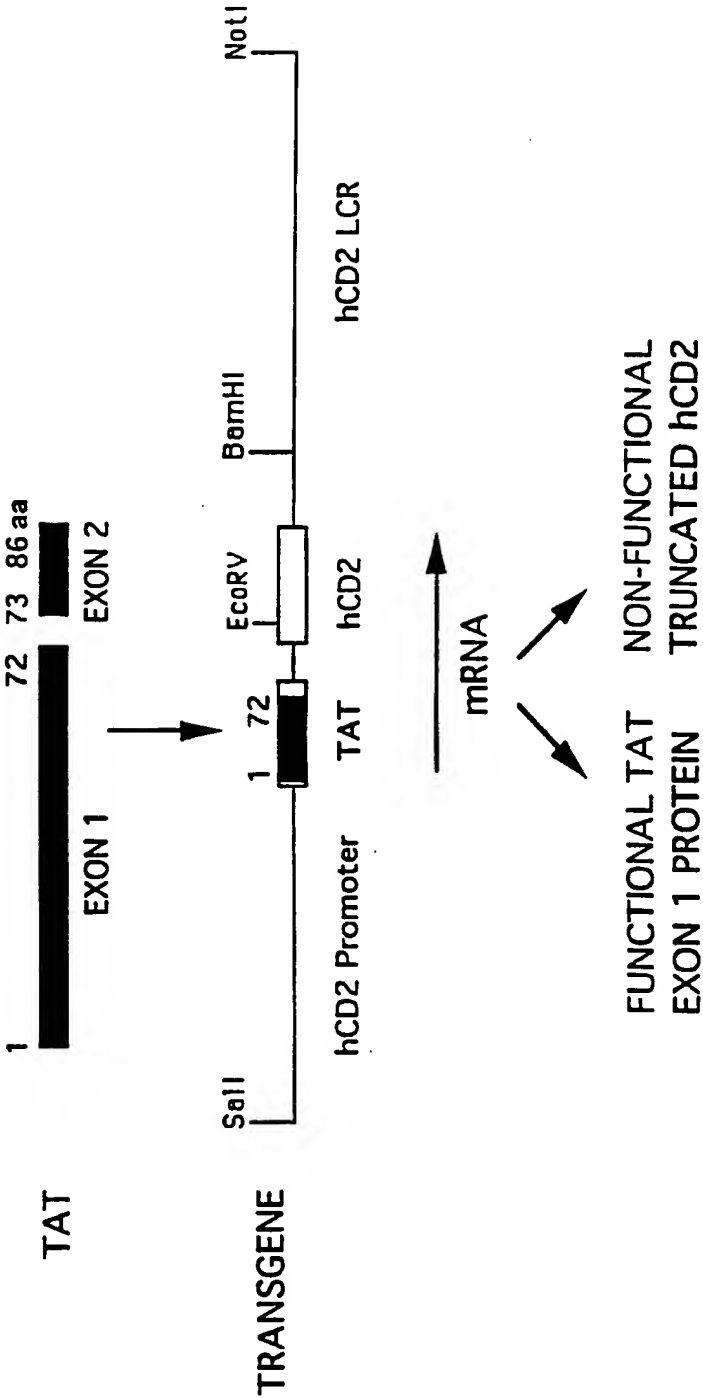
20

21. A cell according to claim 20 which is a stem cell.

22. A cell according to claim 20 which is a T-cell.

23. A cell according to claim 20 which is a macrophage.

FIG. 1



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FIG. 2

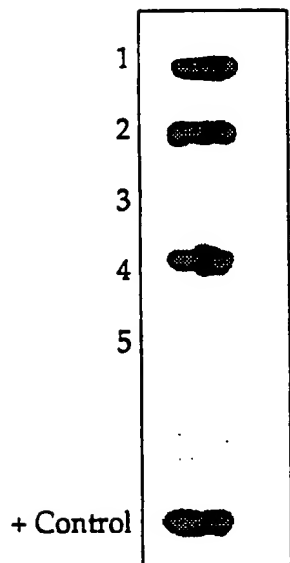


FIG. 3A

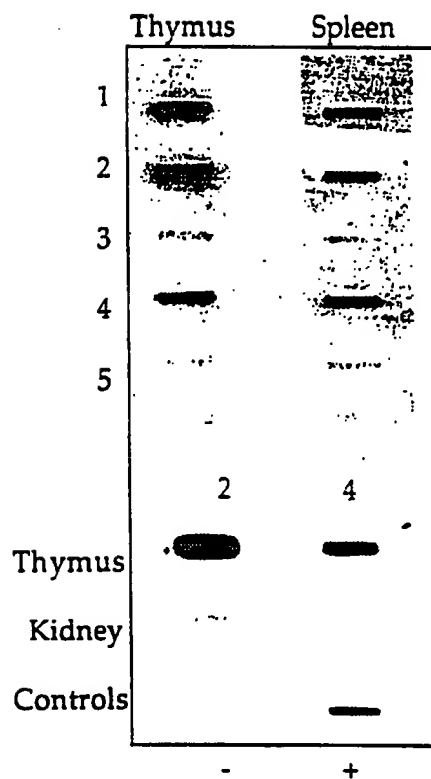


FIG. 3B

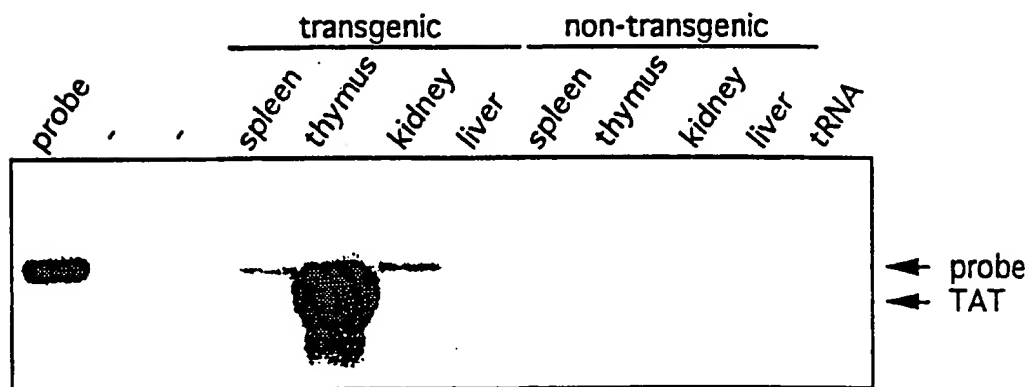


FIG. 4 Thymus

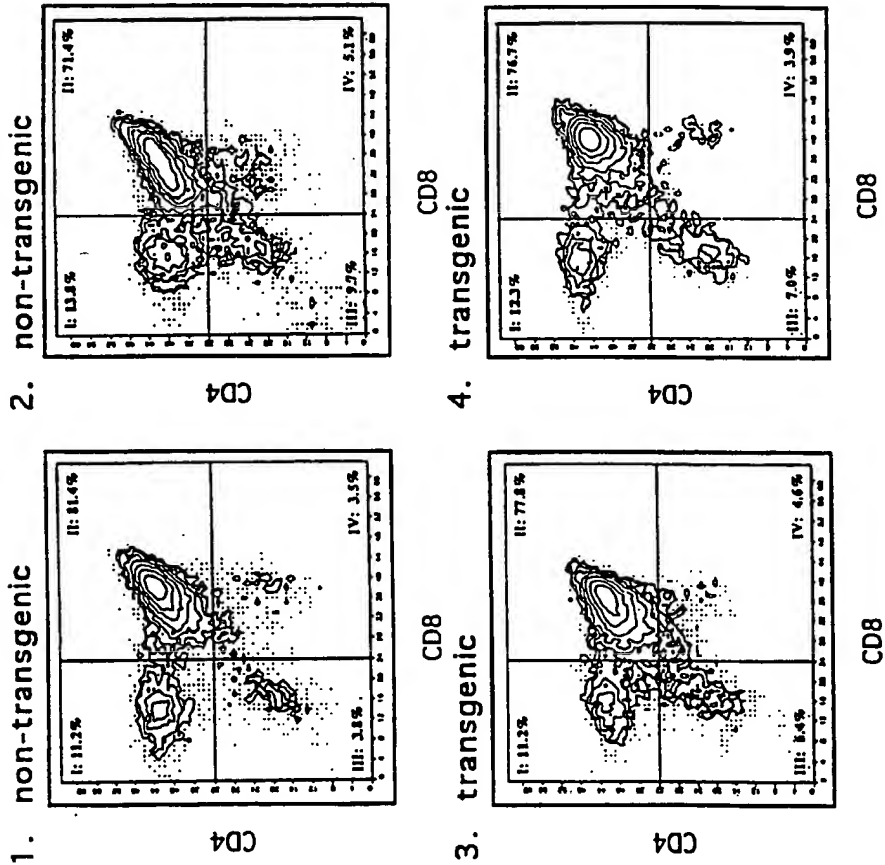
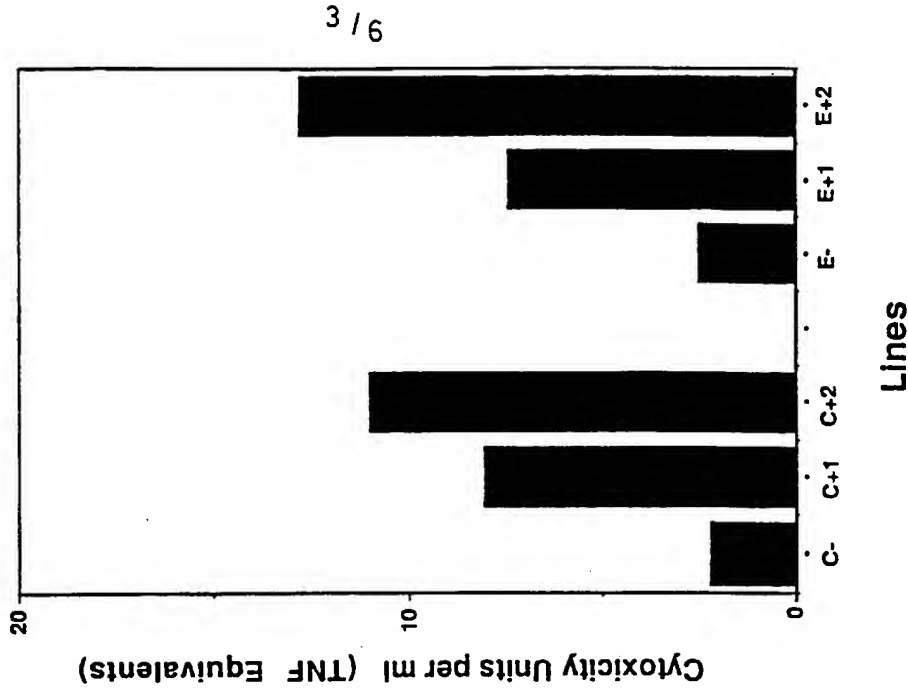
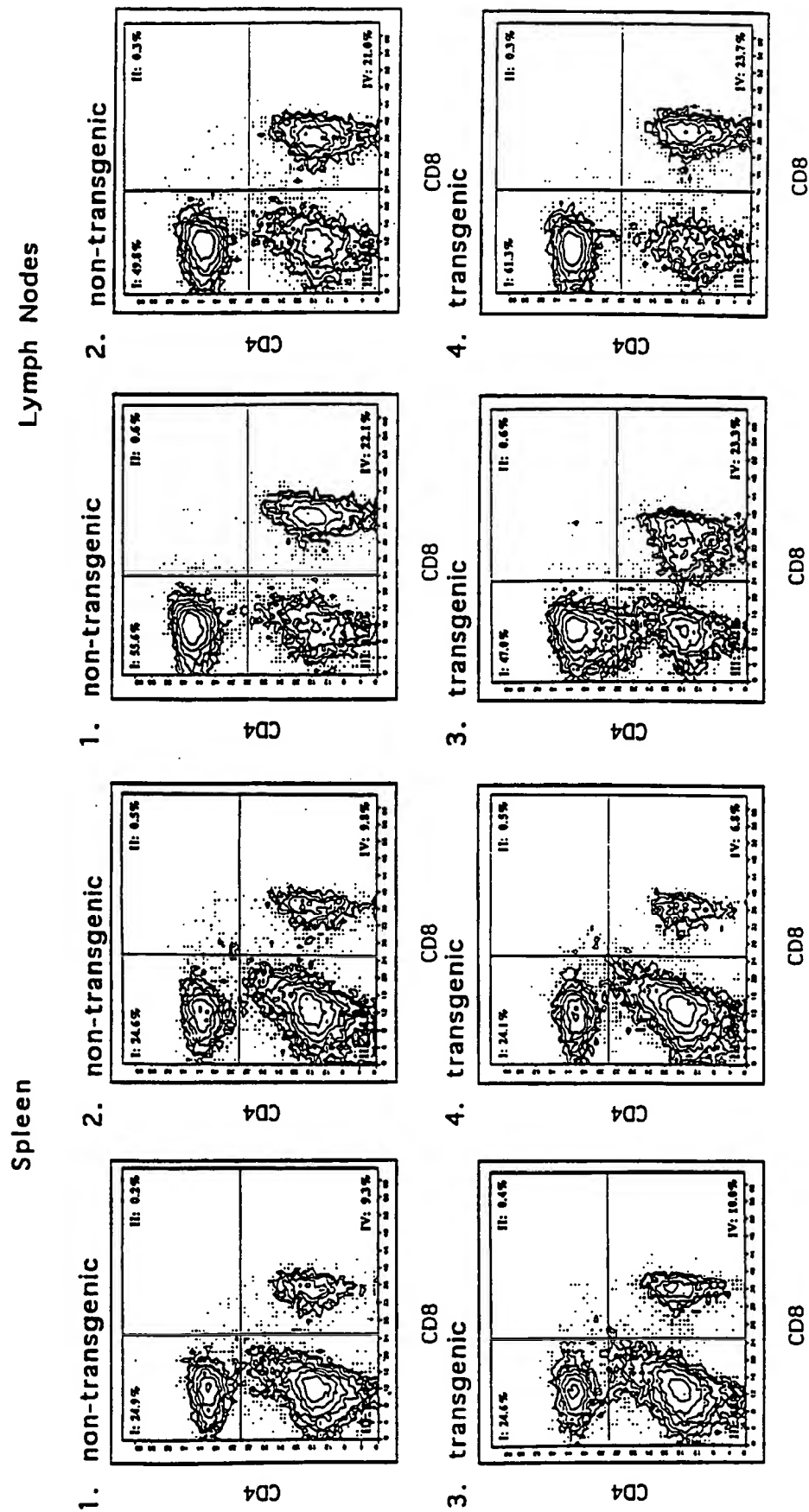


FIG. 6B



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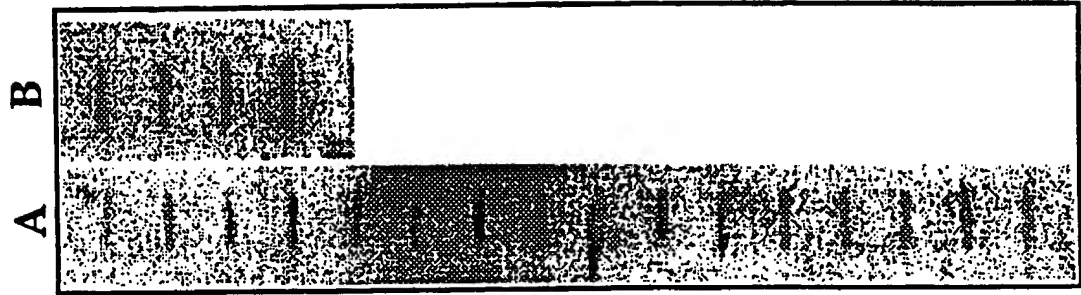
FIG. 5



Copy number controls

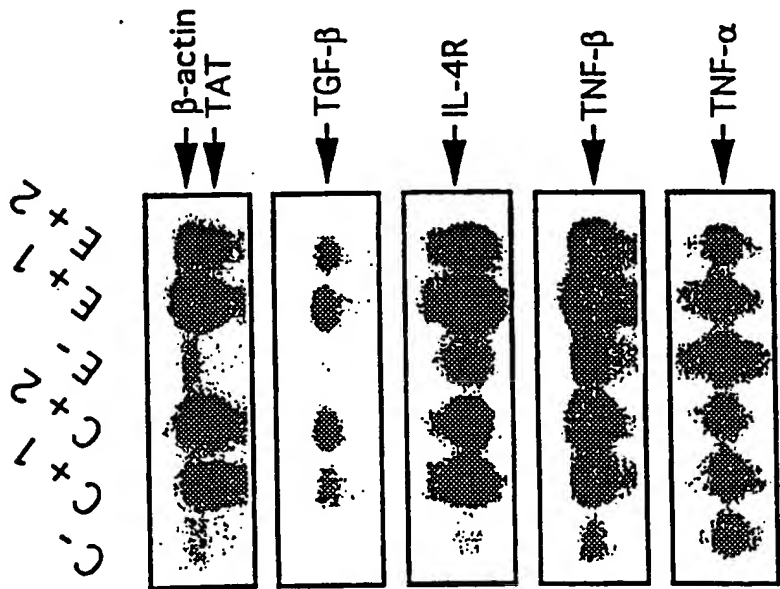
0  
1  
10  
50

FIG. 7



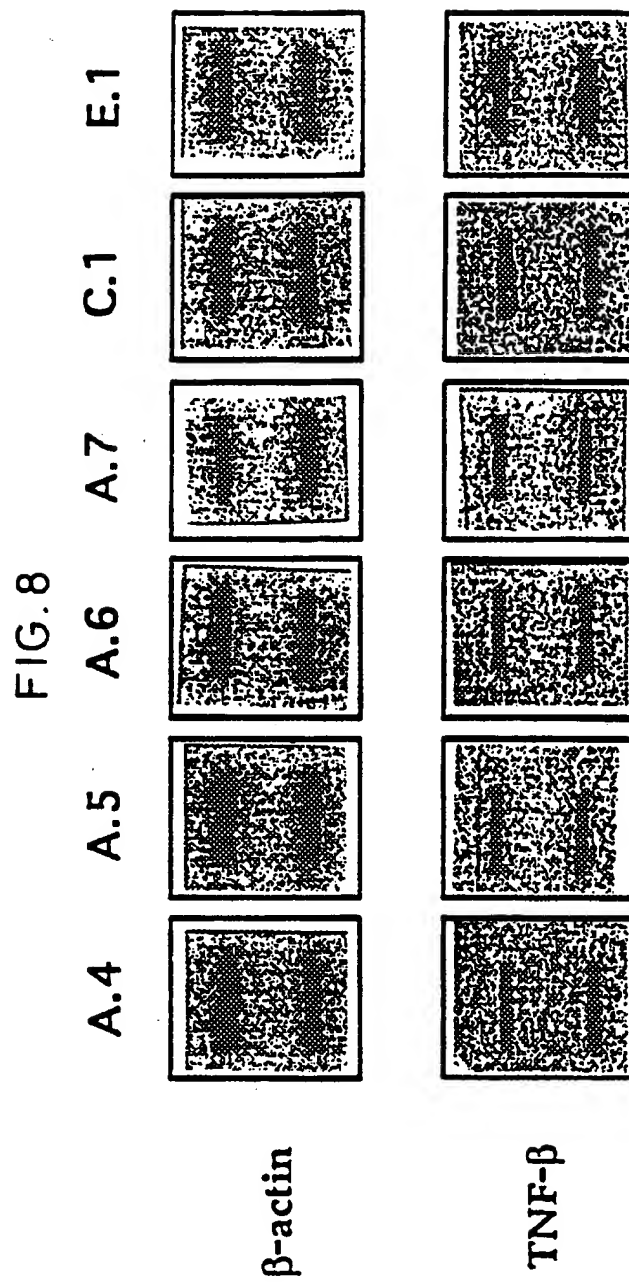
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

FIG. 6A





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## INTERNATIONAL SEARCH REPORT

Intern al Application No

PCT/GB 94/02092

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N15/49 C12N15/12 C12N15/62 A61K48/00  
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NUCLEIC ACIDS RESEARCH., vol.20, no.5, 11 March 1992, ARLINGTON, VIRGINIA US pages 997 - 1003 M. NEEDHAM ET AL. 'LCR/MEL: A versatile system for high-level expression of heterologous proteins in erythroid cells' see abstract see page 998, left column, paragraph 1 see page 999, left column, paragraph 2 - page 1000, left column, paragraph 1 see page 1001, right column, paragraph 2 - page 1002, right column, last paragraph --- -/--	1,4-7, 20,21

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Date of the actual completion of the international search

1 December 1994

Date of mailing of the international search report

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,89 01517 (GROSVELD, FRANKLIN GERARDUS) 23 February 1989 see page 7, paragraph 4 - page 8, paragraph 1 see page 8, paragraph 4 see page 11, paragraph 2 - paragraph 3 see page 12, last paragraph - page 13, paragraph 1 see page 13, last paragraph - page 14, paragraph 1 see page 33, last paragraph - page 34, paragraph 1 ---	1,4-7, 20,21
A	WO,A,91 10453 (THE UNITED STATES OF AMERICA) 25 July 1991  see page 4, line 8 - page 5, line 7 see page 8, line 28 - page 11, line 7 see page 12, line 9 - line 24 ---	1-3,5-8, 10,11, 14-22
E	WO,A,94 21806 (MEDICAL RESEARCH COUNCIL) 29 September 1994  see page 7, line 11 - line 37 see page 8, line 6 - line 22 see page 9, line 28 - page 10, line 13 see page 10, line 35 - page 12, line 10; examples II,III ---	1-8,10, 12,14, 20,22
E	WO,A,94 21111 (MEDICAL RESEARCH COUNCIL) 29 September 1994  see page 4, line 4 - page 6, line 5 see page 6, line 36 - page 7, line 17 see page 12, line 15 - page 14, line 8 see page 18, line 3 - line 19 see page 25, line 26 - page 27, line 13 -----	1-8,11, 13,14, 20,22

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 94/02092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8901517	23-02-89	AU-A- 2137388 EP-A- 0332667 JP-T- 2500802	09-03-89 20-09-89 22-03-90
WO-A-9110453	25-07-91	AU-B- 642959 AU-A- 7074091 EP-A- 0511285 JP-T- 5504255	04-11-93 05-08-91 04-11-92 08-07-93
WO-A-9421806	29-09-94	NONE	
WO-A-9421111	29-09-94	NONE	